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Changes in p-ERK1/2 and p-AKT Expression in Melanoma Lesions after Imatinib Treatment

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Abstract

Response to treatment with imatinib mesylate has been associated in preclinical models with the inhibition of two signaling pathways that promote cellular survival—the phosphatidylinositol 3-kinase (PI3K)/AKT pathway and the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway. We sought to evaluate the extent of inhibition of these two pathways in metastatic melanoma specimens from patients treated with imatinib. Metastatic melanoma tumor samples were obtained before and during the second week of imatinib treatment from patients enrolled in a phase II study. A tissue microarray was constructed using formalin-fixed, paraffin-embedded tissues, and immunohistochemical analysis was performed using standard techniques to detect phosphorylated (p) ERK1/2 and pAKT expression. Of 21 patients who were treated with imatinib, tumor samples adequate for analysis were available both at baseline and during the second week of treatment from 10 patients for pERK1/2 expression and from nine patients for pAKT expression. There was no consistent pattern of change in pAKT or pERK expression after treatment with imatinib. There was no apparent correlation between the clinical benefit of imatinib treatment and changes in pAKT and pERK1/2 expression. A better understanding of the AKT and MAPK pathways is needed to optimize the clinical benefit of targeted therapy, such as imatinib.

Keywords

imatinib; melanoma; pEKR1/2; pAKT; signal transduction pathway

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Introduction

Patients with metastatic melanoma have a poor prognosis, and current treatments have not improved patients' survival. This is due in part to a poor understanding of the biology of melanoma and of how melanoma cells gain the ability to survive and invade tissues at distant sites. Conventional cytotoxic and cytokine therapies are associated with significant toxicities as well as limited activity, so there is a strong impetus to look for more efficacious and specific targeted therapies for melanoma.

Melanoma cells have complicated signal transduction pathways that mediate their survival, proliferation, and invasion. Among these are the mitogen-activated protein kinase (MAPK) and AKT pathways, which have been demonstrated to be constitutively activated in melanoma cell lines [1–3]. Furthermore, the increased expression of phosphorylated AKT (pAKT) in melanoma tumor samples from patients has been associated with disease progression [2, 4].

One possible mechanism of activation of the MAPK and AKT pathways in melanoma cells is the stimulation of upstream kinase receptors, such as C-KIT and platelet-derived growth factor receptors (PDGFRs). These receptors are further activated by growth factors secreted by melanoma cells in both an autocrine and paracrine fashion [5, 6]. When there is an up-regulation of activity at the receptor level, there is a subsequent increase in the activation of the MAPK and AKT pathways.

Imatinib mesylate has been demonstrated to down-regulate the expression of kinase receptors, including C-KIT and PDGFRs in melanoma [7]. It is important to determine whether this decrease in the expression of tyrosine kinase receptors translates into a down-regulation of activity in the MAPK and AKT pathways. A decrease in receptor expression without a corresponding down-regulation of the downstream components of the pathways would likely result in ineffective inhibitory and apoptotic action of imatinib.

We previously conducted a phase II trial of imatinib in patients with metastatic melanoma who underwent tumor biopsies prior to imatinib treatment and then again during their second week of treatment [8]. Among 21 patients were treated with imatinib, one patient (5%) had a partial response, lasting for more than 12 months, and four patients (19%) had a stable disease. In the current studies, we analyzed these tumor samples for expression of phosphorylated extracellular signal-regulated kinase (pERK1/2) and pAKT to determine whether imatinib treatment inhibited the activation of the MAPK and AKT pathways.

Patients and Methods

Patients

A phase II trial testing the clinical efficacy of imatinib was performed at The University of Texas M. D. Anderson Cancer Center between January 2002 and October 2003 [8]. The protocol for this study was approved by the institutional review board of The University of Texas M. D. Anderson Cancer Center. To be eligible for the study, patients must have had stage IV or unresectable stage III melanoma that expressed at least one of the receptor kinases (C-KIT, PDGFR-α or -β, c-ABL, or ARG [Abelson-Related Gene]), as determined by immunohistochemical analysis (IHC) of tumor biopsy specimens at baseline. All patients gave written informed consent before enrollment. Twenty-one patients received 400 mg of imatinib orally twice daily. Repeat tumor biopsy specimens were obtained from patients who gave written consent during the second week of treatment.

Tumor tissue microarray

A tissue microarray was constructed from tumor specimens. First, hematoxylin and eosin (H&E)–stained slides from the available tumor specimens were reviewed. Then, cylindrical tissue cores of 1.0 mm in diameter were punched from formalin-fixed, paraffin-embedded tissue blocks (two cores per biopsy specimen). The cores were then inserted into a 4.5×2×1 cm paraffin block using a manual tissue arrayer (Beecher Instruments) with an edge-to-edge distance of 1 mm. Four-micrometer sections were cut, and one section was stained with H&E to verify the presence of melanoma cells.

IHC analysis

Tissue arrays were stained for pERK1/2 and pAKT using the peroxidase-conjugated avidin-biotin method. Four-micrometer sections were serially cut and mounted on plus-coated slides. The samples were deparaffinized by heating the sections at 60°C for 1 hour on a slide warmer and washing them 3 times in xylene for 3 to 4 minutes. The tissues were then rehydrated in washes of graded alcohol (two of 100% ethanol and one each of 95% and 80% ethanol) for 1 minute each and of phosphate-buffered saline and distilled water for 5 minutes each. The microwave method was used for antigen retrieval: four 2-minute microwave intervals interspersed with 2-minute incubations of the slides outside the microwave. This step was followed by a 30-minute incubation in 0.01 M citrate buffer at pH 6.0 (Antigen Unmasking Solution; Vector Laboratories). Endogenous peroxidase activity was quenched by soaking the slides with 2% sodium hydrogen peroxide in methanol for 20 minutes. After sections were blocked in a universal blocking serum (Vectastain Elite ABC Kit Rabbit IgG; Vector Laboratories) for 30 minutes, they were incubated overnight at 4°C with rabbit anti-human antibody against pERK1/2 (Growth/Proliferation Marker SignalStain Phospho p44/42 MAPK-Thr202/Tyr204 IHC Detection Kit; Cell Signaling Technologies) or with rabbit anti-human antibody against pAKT (Phospho-Akt Ser473 Antibody, IHC Specific [diluted 1:80]; Cell Signaling Technologies). The next day, tissues were incubated with a biotin-labeled secondary antibody and then in avidin/biotinylated enzyme complex (Vectastain Elite ABC Kit Rabbit IgG for pAKT [Vector Laboratories]; Growth/Proliferation Marker SignalStain Phospho p44/42 MAPK-Thr202/Tyr204 IHC Detection Kit for pERK1/2 [Cell Signaling Technologies]) for 30 minutes each. The slides were developed with 3-amino-9-ethylcarbazole (AEC substrate kit SK-4200; Vector Laboratories) and then counterstained with hematoxylin (Vector Laboratories). Positive and negative controls were included.

The stained tissue slides were examined simultaneously by two pathologists and a consensus was reached for the grading of each sample on the basis of the percentage of cells staining positively for pERK1/2 or pAKT. The IHC staining grades were: 0, 5% or fewer positive cells; +1, 6–25% positive cells; +2, 26–75% positive cells; and +3, greater than 75% positive cells.

DNA extraction from paraffin-embedded tumor sections

For *NRAS* and *BRAF* sequencing analysis, sections of the same formalin-fixed, paraffin-embedded melanoma tumors were used as the source of DNA. Between one and four 4-micrometer sections were used per sample, and only sections in which a minimum of 30% of the area consisted of tumor cells were included. Each tumor sample was scraped from the glass slide into xylene using a sterile scalpel blade and then left overnight for deparaffinization. The next day, the tissue pellet was washed twice with 100% ethanol. DNA was extracted using the DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions.

Polymerase chain reaction

Oligonucleotide primers for polymerase chain reaction (PCR) were obtained from Invitrogen Life Technologies. PCR was performed using AccuPrime SuperMix II. PCR primers used are described in Table 1.

DNA sequencing

Sequencing of PCR products was performed by the M. D, Anderson DNA Core Facility using an ABI Prism 3100 DNA genetic analyzer (Applied Biosystems) and Big Dye Terminator v.3.1 chemistry (Applied Biosystems). Forward and reverse DNA strands were sequenced for all samples.

Statistical analysis

The C-KIT and PDGFR-α and -β expressions of the tumors and the clinical data were extracted from our phase II study of imatinib [8]. The possible correlations between changes in pERK1/2 and pAKT expression, clinical response (as defined by the Response Evaluation Criteria in Solid Tumors [RECIST])[9] and clinical benefit (defined as the combination of clinical response and disease stabilization for at least 6 weeks) were examined using Pearson's correlation coefficient, with a *P* value less than 0.05 indicating statistical significance. Similar analyses were performed to evaluate the statistical association between changes in pERK1/2 or pAKT expression and in C-KIT and PDGFR-α and -β expression between baseline and the second week of imatinib treatment.

Results

Of the 21 patients who were treated with imatinib, 13 underwent tumor biopsies during the second week of treatment. We obtained tumor samples adequate for analysis at both baseline and follow-up from 10 patients for pERK1/2 expression and from nine patients for pAKT expression. The samples were adequate for *NRAS* and *BRAF* sequencing analysis in 11 patients.

Table 2 shows the expression of pERK1/2 and pAKT before and during treatment and the status of *NRAS* and *BRAF* mutation. There was no consistent pattern of change in the expression of these proteins after treatment. The expression of pAKT in tumor specimen from the one patient with a clinical response decreased during imatinib treatment. There was no tumoral expression of pEKR1/2 at baseline in the same responder.

There were no obvious correlations between changes in either pERK1/2 or pAKT expression and clinical response or clinical benefit (Tables 3 and 4), although the number of patients who achieved a response (partial response or disease stabilization; four patients) was small.

Discussion

We previously demonstrated that imatinib treatment decreases the expression of its target receptor kinases, such as C-KIT and PDGF receptor-α and -β, in human melanoma specimens [7]. Given the results of these preclinical indicating the down-regulation of receptor kinases, the inactivation of MAPK pathway constituents would be expected in melanoma. However, the results of the present study demonstrate that the MAPK and AKT pathway may not be inactivated by imatinib. Furthermore, baseline expression of pERK1/2 or pAKT does not appear to predict clinical benefit. Regardless, it is interesting to note that the tumor of the one patient with a partial response to imatinib had decreased pAKT expression but no p-MAPK expression during treatment. It could be that imatinib, which

dephosphorylates certain sensitive conformations of receptor kinases, leads to the down-regulation of phosphatidyl-inositol-3-kinase (PI3K)/AKT signaling and that this interruption of AKT signaling might be the main mechanism of action of imatinib in melanoma cells. However, without a large number of clinical responders to analyze, this possible mechanism of action of imatinib in this patient remains only speculative.

A number of studies have examined changes in pERK1/2 expression during imatinib treatment. Dan *et al*. reported that pERK1/2 expression decreased in chronic myelogenous leukemia (CML) cell lines when they were treated with imatinib [10], whereas Li *et al*. demonstrated that pERK1/2 expression in human pancreatic tumor specimens was not altered by imatinib treatment [11]. This discrepancy between studies may have been due, in part, to the differences in staining patterns of cell lines compared with patient samples. However, we can also hypothesize that CML cells, which are primarily driven by *bcr-abl* translocation, have translocation-induced MAPK-dependent cell survival, which is inhibited by imatinib, whereas cells in most solid tumors, such as pancreatic tumors or melanomas, have more complicated signaling pathways.

We found that the AKT pathway was not uniformly inhibited by imatinib, as indicated by the results of pAKT expression. These findings suggest that signal transduction pathways induced by receptor kinases in melanoma may be more complicated than was initially thought. This complexity of signaling pathways may explain the minimal clinical efficacy of imatinib in two phase II trials [8, 12].

Another plausible explanation for the failure of imatinib to inhibit p-EKR1/2 and pAKT in our studies is the presence of *BRAF* or *NRAS* mutations in a large percentage of melanomas. It is estimated that 60–70% and 15–20% of melanomas contain kinase-activating *BRAF* and *NRAS* mutations, respectively [13–15]. In our study, 9 of 11 available tissue specimens contained *NRAS* and/or *BRAF* mutations. It is reasonable to hypothesize that inhibition of upstream receptor kinases by imatinib may not have much of a predictable effect on cell proliferation or survival when downstream proteins, such as *NRAS* and *BRAF*, are dysregulated. In fact, the tumor in the only responder in our trial had neither *NRAS* nor *BRAF* mutations. Future studies of novel inhibitors of receptor kinases may need to select patients whose tumors contain neither *NRAS* nor *BRAF* mutations to achieve the maximum clinical benefit.

The limited effect of imatinib on the inhibition of the MAPK or AKT pathways might be explained by the influence of other signaling pathways that converge onto the MAPK and AKT pathways but act independently of the imatinib-sensitive receptors. For example, Raf kinase inhibitor protein (RKIP) has been demonstrated to inhibit the activation of MEK, a constituent of the MAPK pathway, which is downstream of receptor kinases. RKIP is normally present in many tissues and serves to induce apoptosis and decrease the metastatic potential of cancer cells. As melanoma progresses, RKIP is down-regulated, which indicates that the brake on MAPK activation is released [16, 17]. It could be that reduced RKIP expression will still drive MAPK expression, regardless of MAPK receptor inhibition by imatinib.

In a similar way, insulin-like growth factor receptor (IGFR) activates PI3K, which subsequently activates AKT by phosphorylation. IGFR is not inhibited by imatinib; thus, its possible constitutive activation, as well as that of other pathways not affected by imatinib, could explain our finding of unchanged levels of pERK1/2 and pAKT expression in some patients' tumors, despite treatment with imatinib.

A better understanding of these intricate pathways is necessary for the development of more effective treatments for patients with metastatic melanoma. Although this study has a small number of cases to reach a convincing conclusion, our results highlight the necessity of inhibiting multiple pathways, using combination therapies, to manage the extensive convergence and redundancy of cell signals in metastatic tumors. Current genomic and proteomic studies will likely yield the undiscovered pathways that interact with MAPK and AKT pathways. With that new information, we may be able to personalize therapy with an appropriate combination of targeted drugs and ultimately achieve better clinical efficacy.

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Table 1

Primer sequences used for *NRAS* and *BRAF* sequencing analysis

Gene	Primer Sequence
NRAS exon 2	Forward: 5’-GAACCAAATGGAAGGTCACA-3’ Reverse: 5’-TGGGTAAAGATGATCCGACA-3’
NRAS exon 3	Forward: 5’-GGTGAAACCTGTTTGTTGGA -3’ Reverse: 5’-AACCTAAAACCAACTCTTCCCA-3’
BRAF exon 11	Forward: 5’-TCCCTCTCAGGCATAAGGTAA-3’ Reverse: 5’-CGAACAGTGAATATTTCCTTTGAT-3’
BRAF exon 15	Forward: 5’-TCATAATGCTTGCTCTGATAGGA-3’ Reverse: 5’-GGCCAAAAATTTAATCAGTGGA-3’

Table 2

Phosphorylated extracellular signal-regulated kinase (pERK1/2) and pAKT expression before and during the second week of imatinib treatment

	pERK1/2 expression		pAKT expression		Change in expression						Mutation	
Patient	pERK Baseline	pERK 2nd week	pAKT Baseline	pAKT 2nd week	pERK1/2	pAKT	C-KIT	PDGFR-α	PDGFR-β	Clinical Response	*NRAS*	*BRAF*
1	+2	+1	0	+1	↓	↑	↓	--	↓	PD	N/A	N/A
2	0	0	0	N/A	--	N/A	--	--	↓	SD	WT	V600E
3	+3	+3	0	+3	--	↑	--	--	--	PD	WT	V600E
4	0	0	+2	+1	--	↓	--	--	↓	PR	WT	WT
5	N/A	N/A	+3	N/A	N/A	N/A	↓	↓	↓	PD	WT	V600E
6	+1	+2	+2	0	↑	↓	--	↓	--	PD	WT	V600S
7	0	0	+1	+1	--	--	↑	--	↑	PD	WT	WT
8	0	0	0	+2	--	↑	↑	--	↑	PD	WT	V600E
9	N/A	N/A	+1	0	N/A	↓	↓	↓	↓	PD	Q61R	V600E
10	+3	+2	0	0	↓	--	↓	↓	↓	SD	WT	V600E
11	0	0	+1	+2	--	↑	↑	↓	↓	PD	WT	V600E
12	0	0	N/A	N/A	--	N/A	--	--	--	SD	WT	V600E

Changes in the expression of C-KIT and platelet-derived growth factor receptor (PDGFR)–α and –β were previously published [7]. Numbers indicate percentage of cells staining positively for pERK1/2 or pAKT (0, 0–5%; 1+, 6–25%; 2+, 26–75%; 3+, >75%). ↑, increase in protein expression during treatment; ↓, decrease in protein expression during treatment; --, no change in protein expression during treatment; N/A, no tissue available for analysis; PD, progressive disease; PR, partial response; SD, stable disease; WT, wild type.

Table 3

Correlations among phosphorylated extracellular signal-regulated kinase (pERK1/2) expression, clinical benefit, and changes in the receptor protein kinase expression

Correlation of change in pERK1/2 expression with	Pearson's correlation coefficient	*P*-value
Clinical response (PR)	0.06	0.87
Clinical benefit (PR + SD)	−0.23	0.53
Change in C-KIT expression	0.56	0.09
Change in PDGFR-α expression	−0.12	0.74
Change in PDGFR-β expression	0.40	0.25

Changes in protein expression were categorized as follows: −1, decrease; 0, no change; +1, increase. For clinical response, partial response (PR) was coded as 1 and others as 0. For clinical benefit, PR or stable disease (SD) was coded as 1 and others as 0.

Table 4

Correlation among phosphorylated (p) AKT expression, clinical benefit and changes in receptor protein kinase expression

Correlation of change in pAKT expression with	Pearson's correlation coefficient	*P*-value
Clinical response (PR)	−0.45	0.23
Clinical benefit (PR + SD)	−0.37	0.32
Change in C-KIT expression	0.31	0.42
Change in PDGFR-α expression	0.37	0.33
Change in PDGFR-β expression	0.21	0.59

Changes in protein expression were categorized as follows: −1, decrease; 0, no change; +1, increase. For clinical response, partial response (PR) was coded as 1 and others as 0. For clinical benefit, PR or stable disease (SD) was coded as 1 and others as 0.